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ISOLATION OF β_2-MICROGLOBULIN FROM MINIATURE SWINE SERA

A thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine

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ABSTRACT

β_2-microglobulin ($\beta_2\mu$) has been purified over 1500-fold from the serum of miniature swine poisoned with sodium chromate. Relative to the amount of $\beta_2\mu$ activity in the original serum sample, a 52.2% yield was obtained. A heterologous radioimmunoassay (RIA) inhibition test (using ^{125}I-human $\beta_2\mu$ and goat-anti-rabbit (GAR) $\beta_2\mu$) was used to detect fractions containing swine $\beta_2\mu$ activity in each step of the purification procedure. A procedure of gel chromatography with Sephadex G-75 followed by ion exchange chromatography with DEAE-cellulose was used in the purification. Additional attempts to purify the $\beta_2\mu$ by isoelectric focusing and ion exchange chromatography with CM-cellulose were not successful. Preliminary SDS polyacrylamide gels revealed an 11,000 Dalton putative $\beta_2\mu$ peak free of most contaminants. This $\beta_2\mu$ has been used to immunize a goat, and initial Ouchterlony immunodiffusion tests have shown the ability of the goat-anti-swine $\beta_2\mu$ to form precipitin lines with the purified swine $\beta_2\mu$.

INTRODUCTION

β_2-microglobulin ($\beta_{2\mu}$) is a small protein produced by many cell types, including lymphocytes and cells of epithelial and mesenchymal origin (10,40,47). After synthesis within the cell, it can either be transported to the cell surface, or it can be secreted and is thus found to a small extent in normal body fluids (41). The protein was named on the basis of its small size and its β globulin electrophoretic mobility (49). First isolated from human urine (8), homologues of $\beta_{2\mu}$ have now been isolated and purified from many species, including guinea pig (16), rabbit (6,20,21), mouse (2,39,53), dog (57), cow (31), rat (48), and chicken (66). These homologues of $\beta_{2\mu}$ share many structural characteristics. They are all simple polypeptides of about 100 amino acids, containing no carbohydrate, and have molecular weights between 11,000 and 13,000 Daltons. All except the cow $\beta_{2\mu}$ homologue are monomeric, with one intrachain disulfide bond forming a loop involving 57 amino acids.

The kidney is responsible for much of the catabolism of $\beta_{2\mu}$. Like many small proteins, $\beta_{2\mu}$ is filtered through the glomerulus and is reabsorbed and catabolyzed by proximal tubular cells (3,26). Under normal circumstances, only small amounts of $\beta_{2\mu}$ can be measured in serum and urine, but in cases of renal failure and tubular damage, the serum and urinary levels of $\beta_{2\mu}$ may increase more than a thousandfold (44). Increased serum levels are also seen in lymphoproliferative disorders (9). These cases provide a

source from which $\beta_2\mu$ may be isolated.

The study of $\beta_2\mu$ has become an area of great interest to immunologists because of the resemblance of $\beta_2\mu$ to subunits of immunoglobulins and because it has been found to be a subunit of histocompatibility antigens. The structural similarities among these molecules may imply that they share a similar function, as well as a common evolutionary origin. Therefore it is important to study $\beta_2\mu$ as a model which may clarify the functions of and interrelationships among certain other important cell surface proteins.

Relationship with Immunoglobulins. One of the first observations about $\beta_2\mu$ was that its structure resembled that of the constant regions of an immunoglobulin G (IgG) domain (19,20,22). Both contain about the same number of amino acids and have one intrachain disulfide bond. Each has a compact globular structure with some β-pleated sheet regions. Most importantly, there is considerable amino acid homology between the two, particularly between $\beta_2\mu$ and the C_H3 region of IgG (28% homology). This homology is comparable to the homology among the IgG constant region domains. $\beta_2\mu$ also shows homology to about the same extent with the constant regions of IgM and IgE (19).

In addition to these structural similarities, $\beta_2\mu$ resembles IgG in some of its functions. It can interact with the first component of the complement system like the Fc portion (C_H3 region) of IgG, and when aggregated, can activate complement (43). It is

similar to C_H3 in its ability, when attached to sheep red blood cells, to form rosettes with guinea pig macrophages (43). Like C_H3, $\beta_2\mu$ can also bind to the Fc receptor of lymphocytes (46).

There are some significant differences, however, between $\beta_2\mu$ and immunoglobulin constant regions. While immunoglobulins are made only by plasma cells, $\beta_2\mu$ is made by many nonlymphoid cell types as well as lymphocytes (40). The synthesis of $\beta_2\mu$ is not linked to immunoglobulin synthesis. Another important difference is that antisera to immunoglobulins will not react with $\beta_2\mu$ (8,15).

Inspired by the associations between $\beta_2\mu$ and immunoglobulins, two major hypotheses have been proposed for the evolutionary origin of $\beta_2\mu$. One is that $\beta_2\mu$ is a modern descendant of an immunoglobulin precursor gene, which later duplicates to code for the homologous constant regions of the immunoglobulin molecule (21,22,27,43,46). According to this theory, $\beta_2\mu$ is equivalent to a free C_H3 domain. Evidence supporting this hypothesis includes the presence in invertebrates that lack immunoglobulins, of homologues of $\beta_2\mu$, indicating that $\beta_2\mu$ phylogenetically precedes immunoglobulins. This is also indicated by the presence of $\beta_2\mu$ on nonlymphoid cells. Homology with the constant regions of <u>all</u> classes of immunoglobulins is also evidence that $\beta_2\mu$ occurred first, and immunoglobulins arose from its duplication.

The second major hypothesis about the origin of $\beta_2\mu$ claims that the gene for $\beta_2\mu$ arose after the duplication of the immunoglobulin precursor molecule, from a deletion of a segment in the IgG H chain precursor gene (58). The gene for $\beta_2\mu$ would then include a short segment of the C_H2 region and most of the C_H3 region. This hypothesis is supported by the fact that both structurally and functionally, $\beta_2\mu$ most resembles the C_H3 region, yet it fails to explain why $\beta_2\mu$ would be as similar to IgM and IgE as it is to IgG.

$\beta_2\mu$ is not likely to be the result of a proteolytic cleavage of a larger immunoglobulin for several reasons. There is no fraying at what would be the cleaved end. Also the amino acid sequence does not correspond exactly to a region of any known immunoglobulin. Antisera to the known classes of immunoglobulins do not react with $\beta_2\mu$.

Relationship with Major Histocompatibility Antigens. The serologically determined major histocompatibility antigens (in mice, the K and D region antigens) may be composed of four polypeptide chains, two heavy chains linked by a disulfide bond and two light chains noncovalently bonded to the heavy chains (very similar to immunoglobulin structure). The noncovalent bonding is inferred from the ability to separate the heavy and light chains from each other by low pH without reduction and alkylation (45). This four chain model is not universally accepted, however, and may be due to a solubilization artifact. The alternate structure that has been proposed is a single heavy and light chain noncovalently bonded (61). An association between $\beta_2\mu$ and human histocompatibility (HLA) antigens was first postulated when an 11,000 MW fragment was isolated from human lymphoid cell culture medium

and found to have an identical amino acid composition to human $\beta_2\mu$ (37). Evidence for a structural association between HLA antigens and $\beta_2\mu$ has been shown by co-capping experiments (50,59, 60). Capping is a manifestation of the mobility of membrane proteins. Antibodies to cell surface determinants cause redistribution of these determinants into patches on the cell membrane, followed by energy-dependent migration of these patches to form a cap at either pole of the cell. This aggregate may then be internalized for some proteins, and the membrane protein is regenerated in three to eight hours (56). Treatment of lymphocytes with antiserum against $\beta_2\mu$ leads to co-capping of HLA determinants and $\beta 2\mu$, whereas treatment of the cells with antiserum against HLA antigens induces capping of HLA antigens and only some cell surface $\beta_2\mu$. This occurs on other cell types in addition to lymphocytes (59). This experiment indicates that $\beta_2\mu$ is associated with all serologically determined HLA antigens, but that the number of $\beta_2\mu$ molecules on the cell surface is in excess of the number of HLA antigens (by a six-to-one ratio, as later determined (41)). Therefore, some of the cell-surface $\beta_2\mu$ is associated with HLA antigens, some with other cell surface antigens or receptors (7,41), and up to half of the $\beta 2\mu$ is unassociated with other antigens (33).

Another experiment which shows an association between $\beta_2\mu$ and HLA antigens involves the ability of antisera against $\beta_2\mu$ to precipitate HLA antigens as well as $\beta_2\mu$ (30). After lysis of radiolabelled

lymphocytes with a nonionic detergent, the cell lysates were subjected to immunoprecipitation with anti-$\beta_2\mu$. The precipitates were analyzed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE), giving two peaks of radioactivity, corresponding to 45,000 MW and 11,000 MW (33). The 45,000 MW peak represents the detergent-solubilized HLA antigen without $\beta_2\mu$, while the 11,000 MW peak is $\beta_2\mu$, showing that their association can be disrupted by denaturing conditions.

Through further experiments it was discovered that not only is $\beta_2\mu$ associated with HLA antigens, but $\beta_2\mu$ <u>is</u> the small subunit or "common portion fragment (cpf)" of HLA antigens (30,37,62). This was shown by both structural and functional identity of $\beta_2\mu$ and HLA-cpf. A direct structural comparison was done to determine antigenic and physiochemical properties and amino acid sequence. Antigenic identity was established using a double Ouchterlony technique (62), with antisera to $\beta_2\mu$ and HLA-cpf in two center wells, each encircled by wells containing $\beta_2\mu$ and wells containing HLA-cpf. Lines of identity were obtained throughout. Physiochemical identity was shown by the single band obtained with SDS - PAGE and disc electrophoresis of $\beta_2\mu$ and HLA-cpf molecules, indicating identity of molecular size and ionic charge, respectively (62). Both molecules showed a major dense sharp band at an isoelectric point of 5.0 with isoelectric focusing (IEF), reinforcing the identity of ionic charge. The amino acid sequences were identical for the first 24 amino acids studied (62).

Another experiment showing that $\beta_{2\mu}$ is the small subunit of HLA antigens (45) used papain-solubilized ^{131}I-HLA antigens and ^{125}I-$\beta_{2\mu}$. After reduction and alkylation, fractionation on Sepharose showed ^{131}I activity at peaks corresponding to 33,000 MW and 12,000 MW, and ^{125}I activity at 12,000 MW. The 12,000 MW fraction containing ^{131}I activity from the HLA molecule and ^{125}I activity from $\beta_{2\mu}$ was examined with two dimensional electrophoresis and chromatography, and near identity was found. It was then demonstrated that reconstitution of the HLA molecule was possible with unlabelled 33,000 MW fragment and labelled $\beta_{2\mu}$. When this was done, some of the label was seen associated with the 33,000 MW protein, indicating that $\beta_{2\mu}$ was a part of the reconstituted HLA antigen.

The $\beta_{2\mu}$ subunit of all HLA antigens appears to be invariant, while the larger subunit is the region carrying the antigenic specificity (45). A question not yet answered is whether $\beta_{2\mu}$ modulates the antigenic expression of HLA antigens in a manner similar to the effect of the light chain (L) on the heavy chain (H) of immunoglobulins (51). $\beta_{2\mu}$ may alter the availability of some HLA antigenic determinants (63), or may be required for the surface expression of HLA antigens (17). This is supported by the absence of HLA antigen expression on the surface of cells from the Daudi cell line, a human cell line that cannot make $\beta_{2\mu}$.

Relationship with Mouse Thymus Leukemia (TL) Antigens. Thymus leukemia antigens are cell surface proteins found only on T lymphocytes and lymphoma cells in mice and are coded for by a locus

adjacent to the major histocompatibility complex (H-2). They are very similar in structure to the mouse H-2 antigens. The small invariant subunit has been found to be $\beta_2\mu$ (1,65), shown by migration identical to a $\beta_2\mu$ control on SDS - PAGE. The small subunits are bound noncovalently to two disulfide-linked heavy chains carrying the alloantigenic determinants.

Relationship with Mouse Ss Protein. The Ss protein is a molecule coded for by the S locus of the H-2 complex, to date the only locus of the H-2 which controls a molecule not integrated into the cell surface (47). It has been found to be the murine equivalent of human complement component C4 (23). It also is composed of two subunits, one 23,000 MW and the other 12,000-14,000 MW. The smaller, which is devoid of carbohydrate and contains a single intrachain disulfide bond, may be $\beta_2\mu$ (14). If this is so, it implies important interrelationships among histocompatibility antigens, the complement system, and immunoglobulins.

Relationship with Immune Response-Associated (Ia) Antigens. Ia antigens are coded for by the I region of the mouse H-2 complex. They are found only on limited cell types, including lymphocytes (mainly B cells), spermatozoa, macrophages, and epidermal cells (13). The mixed lymphocyte reaction (MLR) detects differences in I region products, so Ia antigens are described as being "lymphocyte-determined". In the MLR it is mainly B cells which stimulate a response and T cells which respond to allogeneic Ia antigens on the B

cell surface by proliferation. It was found that when the responder T cells were treated first with antisera to $\beta_2\mu$, their proliferative MLR response was inhibited (35). The antibodies may interfere with recognition of the allogeneic Ia antigen by the T cell. This could indicate that they may be bound to an Ia receptor of T cells, which thus may contain $\beta_2\mu$, but this has not been shown conclusively. If the stimulator B cells are treated first with anti-$\beta_2\mu$, T cells in the MLR still respond with normal proliferation, whereas B cell treatment with antisera to Ia antigens leads to inhibition of the T cell proliferative response (47). This seems to indicate that $\beta_2\mu$ is not a subunit of the Ia antigen, since antisera to $\beta_2\mu$ did not appear to block the Ia antigen. Cell surface Ia antigens solubilized from murine lymphocytes do not seem to be associated with $\beta_2\mu$, yet Ia antigens secreted by lymphocytes and obtained without chemical extraction do show an association with $\beta_2\mu$ (3,13). Ia antigens can be identified in serum, associated with high density lipoproteins (HDL) as well as possibly with $\beta_2\mu$ (12,13). It is not yet known if this difference between secreted and cell-bound Ia antigens is real or merely an artifact or a consequence of isolation technique.

Relationship with Allogeneic Effect Factor (AEF). AEF is a soluble immune mediator made by alloantigen-activated T cells which regulates triggering and differentiation of B cells in vitro (2,3). It is not antigen-specific. Like immunoglobulins and histocompatibility antigens, it is made up of two kinds of subunits.

The 40,000 MW subunit contains Ia antigenic determinants, while the 12,000 MW fragment appears identical to $\beta_{2\mu}$(3). AEF can be completely and specifically absorbed by anti-$\beta_{2\mu}$(3). AEF is the first molecule with I region determinants discovered to contain $\beta_{2\mu}$ as part of its structure. This association may indicate that $\beta_{2\mu}$ plays a role in cell-cell interactions.

Relationship with the T Cell Receptor. While the B cell receptor for antigen has been found to be cell surface immunoglobulin, the nature of the T cell receptor has remained a mystery. It has been postulated that $\beta_{2\mu}$, with its similarity to immunoglobulin structure and its linkage with histocompatibility antigens, may be a subunit of the T cell receptor (4,60). To investigate this, the effect of anti-$\beta_{2\mu}$ on responder and effector functions of T cells was observed. Anti-$\beta_{2\mu}$ inhibited the proliferative response of T cells to mitogens (e.g. PHA), to antigens to which the T cells were sensitized (e.g. PPD), and to alloantigens in the MLR. This inhibition could be prevented by addition of pure $\beta_{2\mu}$ which bound to the anti-$\beta_{2\mu}$, preventing its action on T cells. The effector function of T cells is also inhibited by anti-$\beta_{2\mu}$, as shown by the ability of anti-$\beta_{2\mu}$ to block cell-mediated lympholysis (CML). This can be prevented by the addition of purified $\beta_{2\mu}$, to which the anti-$\beta_{2\mu}$ will preferentially bind (4). These findings favor the involvement of $\beta_{2\mu}$ in the T cell receptor. However the invariance or lack of polymorphism of $\beta_{2\mu}$ argues against its role as a T cell receptor subunit, because it would appear to be incapable of mediating

differential receptor activity (4). Also the effects of anti-$\beta_2\mu$ on T cell responder and effector functions may be explained without invoking a necessary link between $\beta_2\mu$ and the T cell receptor. A disturbance of the cell membrane by anti-$\beta_2\mu$ may cause the inhibition of T cell response to stimuli. So the question of whether $\beta_2\mu$ is a part of the T cell receptor remains unsettled.

Studies of $\beta_2\mu$ in many different species give information about the degree of genetic polymorphism or conservation of structure of $\beta_2\mu$ that exists among species. This is important in order to gain insight into the structural, functional, and evolutionary relationships that exist among $\beta_2\mu$ immunoglobulins, histocompatibility antigens, and other cell surface molecules.

While most work done in the past in transplantation biology has utilized the mouse and its well-defined H-2 complex, there are disadvantages to the use of such a small animal. Many animals are required to obtain a quantity of antigen sufficient for biochemical characterization. Also the mouse is not an adequate model for the transplantation of some organs and tissues. It was for these reasons that a large animal model suitable for transplantation studies was sought. Miniature swine were chosen for their size and favorable breeding characteristics (54). They reach sexual maturity at four to six months of age, have an estrous cycle of three weeks, and have a gestation period of about four months. Homozygosity of the major histocompatibility complex was established by inbreeding miniature swine (55).

Analysis of the swine major histocompatibility antigens by SDS electrophoresis has shown a 42,000 MW peak (miniature swine leukocyte antigens or MSLA, comparable to the HLA and the mouse H-2) and an 11,000 MW peak (the putative miniature swine $\beta_2\mu$) (36). The isolation of this miniature swine $\beta_2\mu$, which is the main topic of this paper, was undertaken in order to study different immunological parameters in the pig. Since this animal is a good transplantation model, it was desirable to learn more about the antigens making up its major histocompatibility complex. $\beta_2\mu$ has previously been isolated and purified from several different species.

Human $\beta_2\mu$ was the first to be isolated. It was obtained from the urine of patients with impaired renal tubular function as a result of Wilson's disease or chronic cadmium poisoning (8). The isolation procedure had four main steps: 1) ultrafiltration using dialysis tubing to concentrate the urine; 2) preparative zone electrophoresis in a Pevikon block, first in barbital buffer and then after concentration of the $\beta_2\mu$ peak, in borate buffer (this separates the urinary proteins according to their charge); 3) gel filtration or gel chromatography with a Sephadex G-100 column to separate molecules according to their molecular weight; 4) ion exchange chromatography, first with DEAE-cellulose and then with sulfoethyl Sephadex. Column eluates were analyzed through all these steps by the Folin method to determine total protein, and by Ouchterlony technique or radial immunodiffusion (RID) to determine $\beta_2\mu$ levels. Confirmation of the purity of the isolated $\beta_2\mu$ was obtained by four procedures: 1) Ouchterlony immunodiffusion, which showed that $\beta_2\mu$ present in plasma and urine gave a line of identity with purified $\beta_2\mu$; 2) starch gel electrophoresis, which resulted in one zone

free of contaminants; 3) immunoelectrophoresis (IEP), which showed only one band; and 4) equilibrium ultracentrifugation, which showed a molecular weight of 11,600 for the purified $\beta_2\mu$ molecule.

A simplified method of isolating $\beta_2\mu$ from urine involved the use of affinity chromatography with anti-$\beta_2\mu$ immunoadsorbent columns to bind $\beta_2\mu$ and HLA antigens, separating them from other urinary proteins (42). Another method also using urine as a source of $\beta_2\mu$, proposed the use of an immunoadsorbent column prepared with anti-serum to normal human serum and enriched with monospecific antiserum to glycoproteins (11). There would be very few antibodies to $\beta_2\mu$ and HLA antigens on the column since the concentration of these antigens is quite low in normal sera, so that the high amounts of $\beta_2\mu$ and HLA antigens in the urine of renal patients would pass right through this column. The other proteins also found in the urine of these patients are proteins found normally in sera, and so these would be bound to the column.

A three step procedure using ultrafiltration, gel filtration, and isoelectric focusing (IEF) was also shown to be effective in isolating human $\beta_2\mu$ from urine (64). A flat bed IEF method in poly-acrylamide gel showed high preparative capability. This isolation method utilized radioimmunoassay (RIA) to follow the yield of $\beta_2\mu$ after each step.

$\beta_2\mu$ has also been isolated from guinea pig urine (16), following treatment of the animal with sodium chromate, an agent which leads to

renal tubular damage (5). Two forms of $\beta_2\mu$ were discovered. They were immunologically identical (shown by Ouchterlony lines of identity), but differed in amino acid composition (the minor form had one fewer lysine residue) and isoelectric point. Both forms cross-reacted with human and rabbit $\beta_2\mu$, giving lines of partial identity in Ouchterlony immunodiffusion. Variants of $\beta_2\mu$ may prove to be particularly helpful in determining its biological activity and evolutionary origin. Guinea pig $\beta_2\mu$ was isolated by a procedure involving ultrafiltraion, gel filtration with Sephadex G-100, ion exchange chromatography using DEAE cellulose, zone electrophoresis in borate buffer, and a second gel filtration (16). Total protein and $\beta_2\mu$ activity were identified by absorbance at 280 nm and single radial immunodiffusion, respectively. Purity of the isolated guinea pig $\beta_2\mu$ was confirmed by several methods, including agarose gel electrophoresis, SDS - PAGE, amino acid analysis, IEF, and Ouchterlony immunodiffusion.

Urine from rabbits poisoned with sodium chromate has been another source from which $\beta_2\mu$ has been isolated (6,20,21). The isolation procedure was similar to that used for human and guinea pig $\beta_2\mu$: ultrafiltration, gel chromatography, zone electrophoresis, and DEAE cellulose chromatography. The isolated rabbit $\beta_2\mu$ shows a great similarity with human $\beta_2\mu$ in molecular weight and size, amino acid composition, presence of an intrachain disulfide loop, and electrophoretic mobility. Rabbit $\beta_2\mu$ has also shown homology with rabbit IgG, particularly with the C_H3 region (20). By examining amino acid sequences of $\beta_2\mu$ molecules from humans, rabbits, and dogs, it can be seen that $\beta_2\mu$ structure is highly conserved in evolution, and that $\beta_2\mu$ and C_H2 regions of immunoglobulins show more conservation of

structure across species than the C_H1 and C_H3 regions (21) .

The mouse homologue of human $\beta_2\mu$ has been isolated (2,39, 53) and has been shown to form the small subunit of H-2 antigens coded for by K and D regions (serologically determined), just as human $\beta_2\mu$ has been found to be a part of HLA antigens. Its homology to human $\beta_2\mu$ has been found to be a part of HLA antigens. Its homology to human $\beta_2\mu$ is demonstrated by its ability when on the cell surface, to bind to anti-human $\beta_2\mu$, which in the presence of complement, leads to lysis of the cell (53). It also shows amino acid composition similarity to human $\beta_2\mu$, including presence of an intrachain disulfide bond, and antigenic similarity (39). The amino acid sequence shows much homology with human, dog, and rabbit $\beta_2\mu$ as well as with mouse IgG, particularly the C_H3 region (2). The isolation procedure used for mouse $\beta_2\mu$ involved gel filtration, ion exchange chromatography, and column electrophoresis (39).

$\beta_2\mu$ has been isolated from the urine of dogs with kidney damage induced by surgery and X-rays (52,57). A typical procedure of ultrafiltration, gel filtration, starch gel electrophoresis, and DEAE cellulose chromatography was used. The amino acid sequence of the dog homologue is similar to that of human $\beta_2\mu$, but antisera to the human $\beta_2\mu$ will not precipitate dog $\beta_2\mu$.

Lactollin is a molecule that had been previously isolated and characterized, but was recognized only recently to be the cow homologue of human $\beta_2\mu$ (31). Its amino acid sequence is similar

to that of the $\beta_2\mu$ molecules of other species. Unlike other species, however, it is not found in a monomeric form, but is made up of four noncovalently linked subunits, each of about 12,000 MW.

Thus, in all the different species, the general isolation procedure for $\beta_2\mu$ involved a combination of ultrafiltration, gel filtration, ion exchange chromatography, and zone electrophoresis. The purity of the isolated $\beta_2\mu$ was confirmed by Ouchterlony immunodiffusion, starch gel electrophoresis, or amino acid analysis. Based on this information, a procedure for the isolation of miniature swine $\beta_2\mu$ from serum was devised.

Isolation of $\beta_2\mu$ from miniature swine serum.

MATERIALS AND METHODS

Animals. A miniature swine from the inbred herd maintained at the National Institutes of Health Animal Center, Poolesville, Maryland, was poisoned with 20 mg/kg sodium chromate injected subcutaneously, as previously described (5). In rabbits, the maximum induced proteinuria occurs three to five days post-injection (20), so urine (via a catheter) and a large sample of blood were collected from the pig daily, from day three until day seven, when the pig was exsanguinated. Sodium azide was added to the urine samples which were then kept frozen at -20°C. The blood samples were centrifuged and the sera were also stored at -20°C.

Radioimmunoassay (RIA). In order to isolate miniature swine $\beta_2\mu$ it was necessary to have an assay capable of detecting $\beta_2\mu$ activity in column fractions from each step of the isolation procedure. It had been shown that a heterologous radioimmunoassay can be used to detect homologues of $\beta_2\mu$ in sera from various vertebrate species (28). There is sufficient homology among the $\beta_2\mu$ molecules of different species for the $\beta_2\mu$ of one species to bind to the antisera against the $\beta_2\mu$ of another species. In the system described (28), significant binding (about 60%) occurred between ^{125}I-human $\beta_2\mu$ and goat-anti-rabbit (GAR) $\beta_2\mu$. This binding could be inhibited by unlabelled $\beta_2\mu$ molecules from many species, including the pig, which showed 81%

inhibition (28). A homologous system using binding between labelled $\beta_2\mu$ and antisera to $\beta_2\mu$ from the same species, was not inhibited by $\beta_2\mu$ from another species, so this could not be used.

The heterologous radioimmunoassay using inhibition of binding between ^{125}I-human $\beta_2\mu$ and GAR $\beta_2\mu$ was used in this isolation procedure. The GAR $\beta_2\mu$ was obtained from Doctor Thomas Kindt (National Institute of Allergy and Infectious Disease, National Institutes of Health), and the IgG fraction of it was coupled to Sepharose for use in the assay by Doctor Stuart Rudikoff (Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health). Purified human $\beta_2\mu$ was kindly provided by Doctor Howard Grey (National Jewish Hospital Research Center, Denver, Colorado). It was labelled with ^{125}I by the lactoperoxidase-Sepharose gel iodination method as previously described (24).

An RIA binding assay was set up in duplicate using a constant amount of ^{125}I-human $\beta_2\mu$ and several different quantities of Sepharose-bound GAR $\beta_2\mu$. Before use, the ^{125}I-human $\beta_2\mu$ was diluted so that each 100 µl contained about 20 ng of protein (as estimated by absorbance at 280 nm) and 50,000 counts of ^{125}I. The other components of this solution were: 1) emulphogene, an emulsifier added in a ratio of about 25 to 30 µl per ml of solution; 2)phosphate-buffered saline (PBS), pH 7, with 0.01% NaN_3 and 1% bovine serum albumin (BSA); 3) ^{57}Cobalt, about 500,000 counts per ml of solution. This was added as a volume control to allow calculation of the bound ^{125}I-human $\beta_2\mu$ without having to wash all the free ^{125}I-human $\beta_2\mu$ from the precipitate (29).

Binding and inhibition assays were both set up in Brinkmann microfuge tubes, each brought to a final volume of 300 ul. In both of these assays a background tube containing only the PBS-BSA buffer and an antigen control tube containing only ^{125}I-human $\beta_2\mu$ and the buffer were set up. A control for nonspecific antibody binding was also made, using ^{125}I-human $\beta_2\mu$ and another Sepharose-bound antibody. All tubes were mixed continuously overnight at 4^{o}C to optimize binding. Then after centrifuging for one minute in a Brinkmann microfuge, about fifty percent of the supernatent of each tube except the antigen control was removed. The antigen control tube was counted in its entirety, to give the maximum ^{125}I counts possible. Samples were counted in a gamma counter capable of counting two channels, ^{125}I and ^{57}Co, for each tube.

For the inhibition RIA assay, an amount of GAR $\beta_2\mu$ was chosen that resulted in fifty percent binding. In addition to the controls used for the binding assay, a zero-inhibition or 100% binding control was set up, containing only the ^{125}I-human $\beta_2\mu$ and Sepharose-bound GAR $\beta_2\mu$. All experimental samples contained these same quantities of ^{125}I-human $\beta_2\mu$ and GAR $\beta_2\mu$ plus an inhibitor which could be serum, urine, eluate from a column, or anything containing $\beta_2\mu$. Unlabelled human $\beta_2\mu$ was used as a standard inhibitor in several different dilutions each time the assay was run. Samples with a high percent inhibition were diluted in the PBS-BSA buffer and titered to find the maximum dilution that would still show inhibition.

The percent binding was calculated by the formula:

$\%B = 100\left[\frac{Ea - eA}{A(a - e)}\right]$ where E = ^{125}I channel of the experimental tube, e = ^{57}Co channel of the experimental tube, A = ^{125}I channel of the antigen control, and a = ^{57}Co channel of antigen control (29). Additional corrections were made for background counts. The percent inhibition was calculated by the formula:

$$\%I = \frac{\%B \text{ of zero inhibition control} - \%B \text{ of sample inhibitor}}{\%B \text{ of zero inhibition control}}$$

where %B is percent binding calculated as above (32).

Gel Chromatography. 100 ml aliquots of serum from the poisoned pig were centrifuged at 15,000 rpm for 15 minutes to remove any clumps and then chromatographed on a Sephadex G-75 column (200 x 5 cm) in borate buffer (0.2 M boric acid, 0.16 M NaCl, pH 8.0). At a flow rate of 25 ml per hour, each 100 ml sample took about six to seven days to be eluted from the column. About 270 18 ml fractions were collected. Absorbance at 280 nm was used as an indication of total protein. Fractions showing $\beta_2\mu$ activity as assayed by their ability to inhibit ^{125}I-human $\beta_2\mu$ -GAR $\beta_2\mu$ binding in the RIA, were pooled. Pools were concentrated by ultrafiltration in an Amicon concentrator with a UM-2 filter, retaining proteins with a molecular weight greater than 2000 Daltons. The pass-through was assayed for $\beta_2\mu$ activity, to be sure that $\beta_2\mu$ was not being lost through the filter. Initially a UM-10 filter had been used, but there was too great a loss of $\beta_2\mu$ activity with this pore size.

Isoelectric focusing (IEF). 1.0 ml of the UM-2 concentrate was dialyzed overnight into glycine-HCl buffer to decrease the salt concentration, using Spectropor 3 tubing, which retains molecules greater than 3500 MW. This sample was then loaded on a 110 ml preparative IEF column run at 4°C for 24 hours. Carrier ampholyte solutions of pH range from 3 to 10 were used. A solution of 50% sucrose was used to form a density gradient to stabilize the protein zones. 45 fractions of two mls each were analyzed for pH, for total protein by absorbance at 280 nm, and for $\beta_2\mu$ activity by RIA.

Ion Exchange Chromatography. Another aliquot of the UM-2 concentrate of serum $\beta_2\mu$ was dialyzed overnight into 0.01 M Tris, pH 8.6, and chromatographed on a 9 ml DEAE column equilibrated in the same buffer. A gradient elution device was connected to the column, with a 60 ml mixing chamber containing the equilibration buffer, and a 60 ml reservoir of equilibration buffer containing 0.2 M NaCl. After the first 10 tubes of about 2 ml each were collected, elution with the linear salt gradient was started. About 50 more tubes were collected and all were assayed for $\beta_2\mu$ activity by RIA. Those with $\beta_2\mu$ activity were pooled and then concentrated by ultrafiltration using the Amicon with a UM-2 filter. This concentrate was then equilibrated with sodium acetate 0.01 M, pH 5.6, by dialysis overnight using Spectropor 3 (3500) tubing, and was placed on a CM cellulose column equilibrated in the same buffer. It was eluted with a linear salt gradient made by mixing the 0.01M sodium acetate buffer with equilibration buffer containing 0.2M NaCl.

RESULTS

Radioimmunoassay. ^{125}I-labelling of the human $\beta_2\mu$ to be used in the RIA resulted in a specific activity of 3010 cpm/ng. The RIA binding assay using the ^{125}I-human $\beta_2\mu$ and GAR $\beta_2\mu$ antiserum showed binding that plateaued at about 80 percent (Figure 1). Based on this binding curve, the RIA inhibition assay was set up using the ratio of GAR $\beta_2\mu$ to ^{125}I-human $\beta_2\mu$ that gave 50 percent binding, as determined from the binding curve. Different concentrations of unlabelled human $\beta_2\mu$ were used as standard inhibitors of binding, and Figure 2 shows the inhibition curve obtained. A plateau of inhibition occurred at about 65 percent. From the percent inhibition observed in sera and urines, an estimate of their $\beta_2\mu$ concentration could be made using this standard curve. This assumes that unlabelled human $\beta_2\mu$ and pig $\beta_2\mu$ bind to ^{125}I-human $\beta_2\mu$ to the same degree. In the past (28), human and pig sera have both shown about 80 percent inhibition of the GAR $\beta_2\mu$ - ^{125}I-human $\beta_2\mu$ heterologous system, so this assumption is probably valid. By this estimation the concentration of $\beta_2\mu$ in normal pig serum was 1.46 ug/ml. The serum from a pig poisoned with sodium chromate contained about 14.64 ug/ml, about a tenfold increase over the normal pig serum.

With this RIA inhibition assay established as a reliable method of detecting and estimating the quantity of $\beta_2\mu$ activity, it was then used to determine fractions with $\beta_2\mu$ activity in each step of the isolation procedure. The isolation described here was only done with the serum from the poisoned pig, but the urine will be dealt with in the same manner.

Gel Chromatography on Sephadex G-75. Serum from the pig poisoned with sodium chromate was chromatographed to separate out small molecular weight molecules like $\beta_2\mu$ (about 12,000 Daltons) from the larger serum proteins. Figure 3 shows this separation, with the major peak in OD_{280}, presumably the major serum proteins, eluted from the column first, followed by and not overlapping with the peak having the greatest $\beta_2\mu$ activity. The first peak of $\beta_2\mu$ activity may represent a small amount of $\beta_2\mu$ that is still bound to histocompatibility antigens, thus eluted with the higher molecular weight proteins. The fractions after the major $\beta_2\mu$ peak that show $\beta_2\mu$ activity may contain smaller molecular weight breakdown products of $\beta_2\mu$ with less RIA inhibitory activity. Two subsequent serum separations on this Sephadex G-75 column showed that absorbance at 280 nm and $\beta_2\mu$ activity occurred in approximately the same fractions as in the first separation.

Isoelectric Focusing. After pooling the fractions with the greatest $\beta_2\mu$ activity, as shown in Figure 3, a portion of the concentrated pool was analyzed by isoelectric focusing in an attempt to separate $\beta_2\mu$ from molecules of the same size but of a different charge or isoelectric point. Although the pH gradient was good, the $\beta_2\mu$ activity did not focus in one fraction, but was scattered over a wide pH range (Figure 4). To determine whether the RIA might be showing nonspecific inhibition by sucrose or by some other molecule used in the procedure, the fractions were dialyzed. This resulted in $\beta_2\mu$ activity that was somewhat more focused, but still quite diffuse. Therefore this IEF procedure was abandoned, rather than using up more sample to repeat it.

Ion Exchange Chromatography. It was decided instead to separate the Sephadex G-75 concentrated pool by charge with a DEAE-cellulose column. The majority of the $\beta_2\mu$ did not pass straight through the positively charged DEAE column (pH 8.6) but rather, showed binding to the column, indicating the negative charge of pig $\beta_2\mu$ at this pH. It was eluted only after the salt gradient was started through the column. Most of the $\beta_2\mu$ activity appeared in one distinct peak, pooled as shown in Figure 5. This pool was then concentrated and applied to a CM cellulose column (pH 5.6). This step was not successful. Fractions eluted from the column showed little $\beta_2\mu$ activity, probably due to aggregation and failure to be eluted at the acid pH.

A summary of results from the successful isolation steps was compiled in Table I. The total amount of $\beta_2\mu$ resulting from each purification step (as determined by RIA inhibition) was compared to the total amount of $\beta_2\mu$ in the original serum sample to obtain a percent yield for each step. Also the ratio of total $\beta_2\mu$ to total protein as calculated by absorbance at 280 nm, in the original serum was compared to this ratio in each subsequent purification step, to determine how manyfold a purification was achieved. The net result of the isolation procedure up to and including the DEAE-cellulose ion exchange step was a 52.2% yield of $\beta_2\mu$ with respect to the amount of $\beta_2\mu$ activity in the original serum, and a greater than 1500-fold purification.

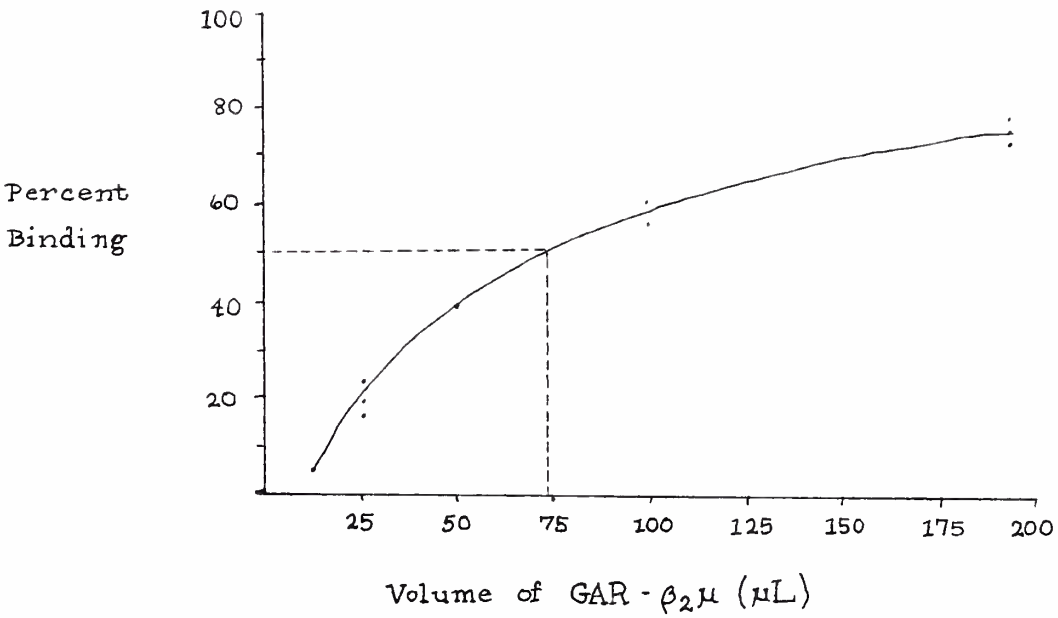

Figure 1. Radioimmunoassay binding curve. Concentration of GAR $\beta_2\mu$ giving 50 percent binding is shown. (20 ng. of ^{125}I-human $\beta_2\mu$ were used in each microfuge tube).

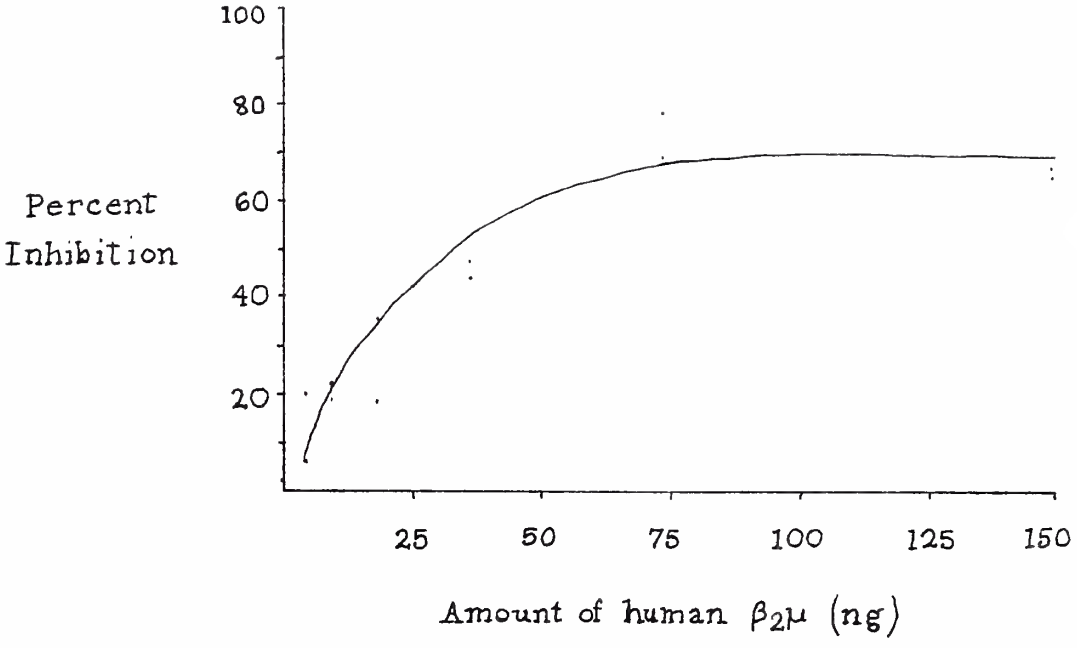

Figure 2. Radioimmunoassay inhibition curve. Standard unlabelled human $\beta_2\mu$ was used as inhibitor of a heterologous system of 20 ng ^{125}I-human $\beta_2\mu$ and 75 µl goat anti-rabbit (GAR) $\beta_2\mu$ per microfuge tube.

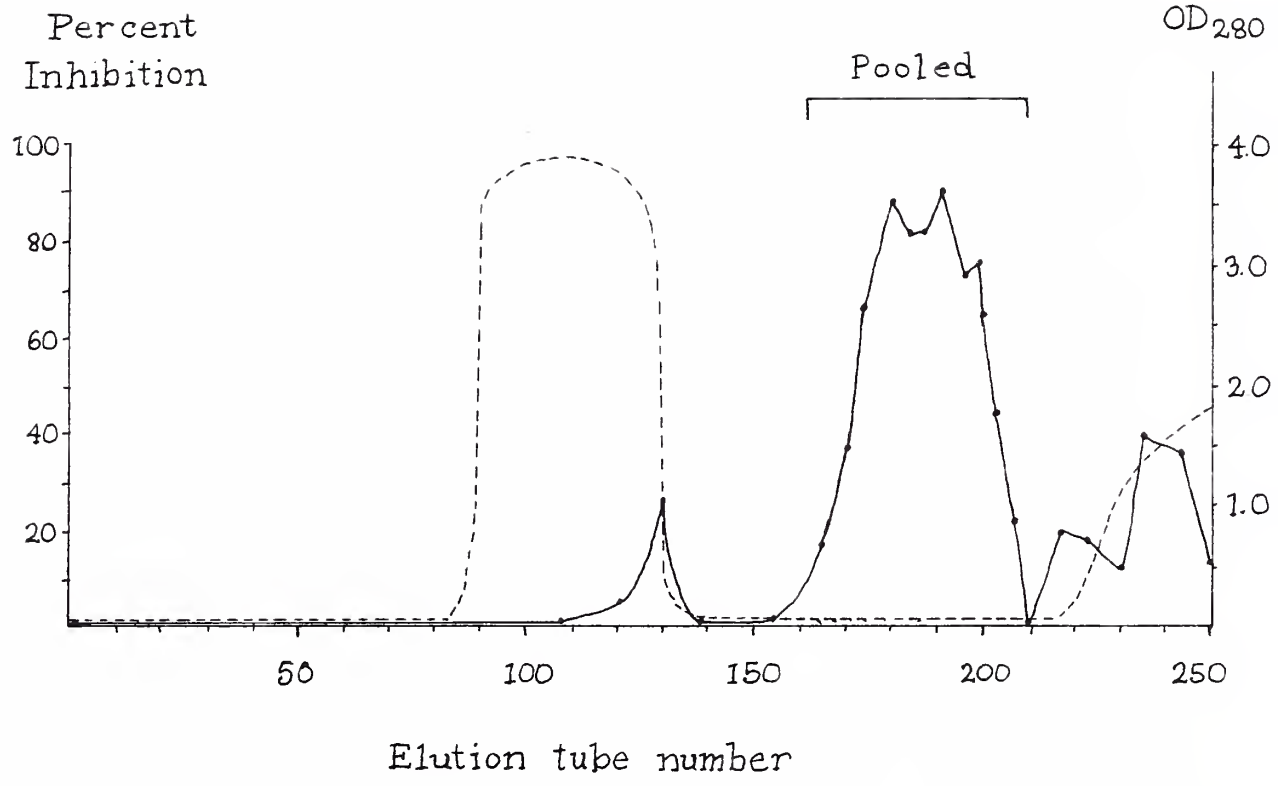

Figure 3. Sephadex G-75 gel filtration of pig serum. A 200 x 5 cm column was run in 0.2m borate buffer, pH 8.0. OD_{280} (---) was used as a measure of total protein, and percent inhibition of heterologous (^{125}I-human $\beta_{2\mu}$ -GAR $\beta_{2\mu}$) RIA binding (——) was used as an index of $\beta_{2\mu}$ activity. Fractions with greatest $\beta_{2\mu}$ activity were pooled as indicated.

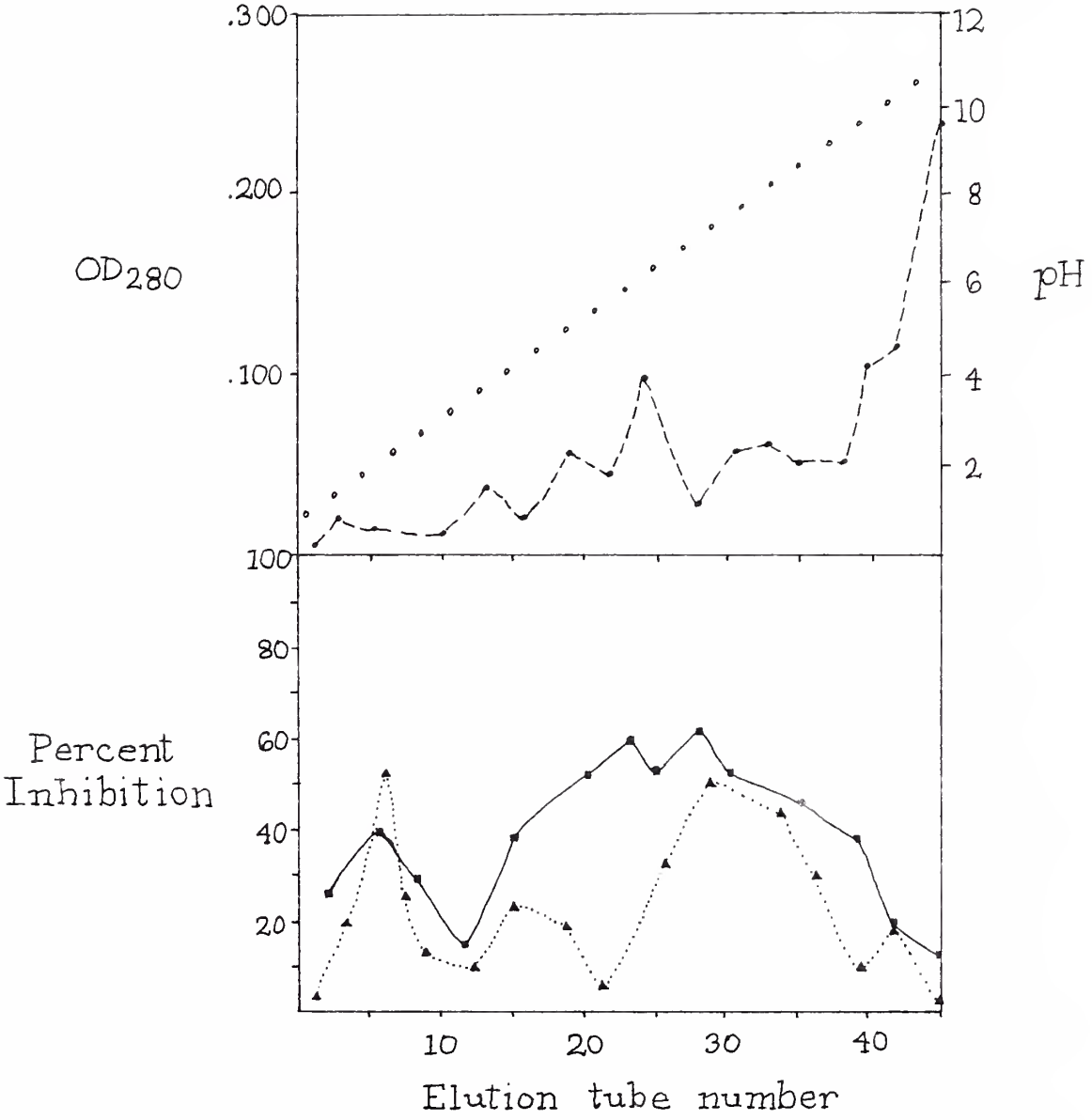

Figure 4. Isoelectric focusing. The concentrated $\beta_{2\mu}$ peak from the Sephadex G-75 column was applied to a 110 ml preparative IEF column. A gradient from pH3 to pH10 (•••••) was formed, stabilized by a 50% sucrose gradient. The OD_{280}(•—•) of the fractions is shown on the upper part of the graph. On the lower part, the $\beta_{2\mu}$ activity (as determined by percent inhibition of a heterologous RIA inhibition system) is shown for the undialyzed (•——•) and the dialyzed (▲·····▲) fractions.

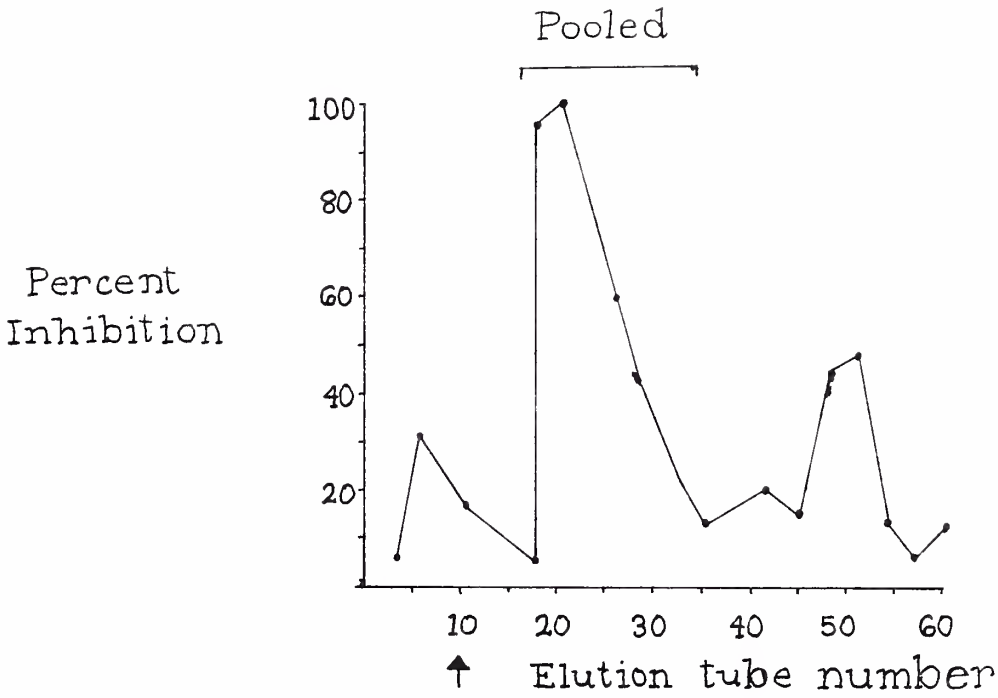

Figure 5. Ion exchange chromatography using DEAE cellulose. Elution diagram of the concentrated $\beta_2\mu$ peak (from the Sephadex G-75 column) applied to a DEAE cellulose column (0.01 M Tris, pH 8.6). Arrow marks the start of elution with 0.2 M NaCl.

TABLE I. Purification of Miniature Swine $\beta_{2\mu}$ -microglobulin

Step	Total protein concentr. (mg/ml)[a]	Total prot. in sample (mg)	Volume (ml)	$\beta_{2\mu}$ concentr. (ug/ml)[b]	$\beta_{2\mu}$ total in sample (mg)	% Yield	Purification (fold)
Unconcentrated serum	88.990	8899.00	100.0	14.64	1.464	100.0	1
Sephadex G-75 $\beta_{2\mu}$ pool	0.047	32.83	703.0	1.83	1.286	87.8	238
Concentrated G-75 pool	1.640	17.22	10.5	124.40	1.307	89.3	461
Concentrated DEAE pool	0.453	3.07	6.8	112.90	0.765	52.2	1514

[a] Calculated from OD_{280}, using 1.69 as an extinction coefficient (previously determined for human $\beta_{2\mu}$ (8)).

[b] Calculated from RIA inhibitory activity.

DISCUSSION

The isolation of miniature swine $\beta_{2\mu}$ was undertaken in order to further investigate the structures of $\beta_{2\mu}$ and the histocompatibility antigens and their homology with the comparable molecules in other species. A remarkable retention of structural homology in the $\beta_{2\mu}$ molecule has been shown in the many species studied thus far. This is reflected in immunologic cross-reactions that have been detected between mouse and rabbit $\beta_{2\mu}$ and between mouse and human $\beta_{2\mu}$ by RIA assays (38), and between rabbit and human $\beta_{2\mu}$ by Ouchterlony analysis (6). This cross-reactivity was utilized in the present study, where miniature swine $\beta_{2\mu}$ showed inhibition of a heterologous RIA system of goat-anti-human $\beta_{2\mu}$ and human $\beta_{2\mu}$. This inhibition assay provided a means of following the progress of the miniature swine $\beta_{2\mu}$ isolation.

Whereas most other studies have used urine of poisoned animals or of humans with kidney damage as a source of $\beta_{2\mu}$, this study was done using serum of a poisoned pig, which also showed high levels of $\beta_{2\mu}$ activity. (The urine will be used in the same manner, to get the maximal amount of $\beta_{2\mu}$ available from each animal and provide more $\beta_{2\mu}$ for chemical characterization). Isolation steps were basically similar to those used in purification of human, guinea pig, rabbit, and mouse $\beta_{2\mu}$. Most of these used ultrafiltration, gel chromatography, and some form of ion exchange chromatography. Isoelectric focusing had only been used in one of the human $\beta_{2\mu}$ isolation procedures (64), but did not result in the same success in the present isolation scheme with the pig sera. The failure of the $\beta_{2\mu}$ to focus may have been due to nonspecific binding of $\beta_{2\mu}$ to proteins of different isoelectric points.

Various attempts to further purify the pig $\beta_2\mu$ by CM cellulose or gel filtration at acidic pH have been unsuccessful, possibly due to insolubility of the pig $\beta_2\mu$ at this pH. Instead, following the DEAE-cellulose step, an additional gel chromatography step using Biogel-P10 at a basic pH is currently being carried out. Preliminary SDS gel electrophoresis tests on the concentrated DEAE pool using a method previously described (18,34) show a 11,500 MW peak free of most contaminants. This material is now being assayed for purity by amino acid sequencing. When sequenced, it can be compared with the $\beta_2\mu$ molecules of other species to determine the degree of homology. Another use for the purified $\beta_2\mu$ is in reassociation studies with purified MSLA antigens, to see if full alloantigenic activity can be recovered.

Studies are being carried out now using the miniature swine $\beta_2\mu$ isolated as described above to make an antiserum to swine $\beta_2\mu$. A goat was injected intramuscularly at four sites with a total dose of 100-200 ug of purified swine $\beta_2\mu$ (from the concentrated DEAE pool) in complete Freund's adjuvant and was boosted in the same manner at three weeks. Sera from weekly bleeds of the goat show precipitin bands with the purified swine $\beta_2\mu$ in Ouchterlony immunodiffusion and were cytotoxic to pig lymphocytes. Preliminary SDS polyacrylamide gels with radiolabelled miniature swine lymphocyte antigens indicate that the goat-anti-swine $\beta_2\mu$ antisera appears to precipitate histocompatibility antigens.

Further experiments will attempt to use the antiserum to swine $\beta_2\mu$ to make an anti-$\beta_2\mu$ immunoadsorbent column for use in the isolation of MSLA antigens. There have been problems in the past with the use of anti-$\beta_2\mu$ immunoadsorbent columns as a means of isolating HLA antigens, based on difficulty in elution of the antigens from the anti-$\beta_2\mu$. The noncovalent binding between $\beta_2\mu$ and the histocompatibility antigens was readily disrupted in solution, but more stringent conditions were required to elute the antigen from the anti-$\beta_2\mu$ column. This may have been due to aggregation of HLA molecules, forming a network on the column which was difficult to elute. With 0.1% SDS at pH 7, however, 40–50% of the HLA antigenic activity put on the column was able to be eluted (25). Others have shown effective elution of HLA antigen from an anti-$\beta_2\mu$ immunoadsorbent column with excess $\beta_2\mu$ or with 0.05 M glycine/HCl buffer (pH 2.4). These methods gave 60 to 90 percent recovery of alloantigenic activity (54). Anti-$\beta_2\mu$ immunoadsorbent columns may avoid the need for a whole series of fractionations and may yield a product of higher purity than multistep procedures, based on the specific binding of the $\beta_2\mu$ moiety of the MSLA antigens to the anti-$\beta_2\mu$ on the column. Anti-$\beta_2\mu$ can also be used in immunoprecipitation gels, to attempt to precipitate out $\beta_2\mu$ and MSLA antigens and any other cell surface molecules with which $\beta_2\mu$ may be associated.

When isolation and purification of the MSLA antigens is achieved, these antigens can be sequenced and compared with immunoglobulin structures and with H-2 and HLA antigens to establish homology. Further studies will look at homology within the three different homozygous MHC types, AA, CC, and DD, that have been established in miniature

swine (55). Then the homology of AA, CC, and DD antigens with histocompatibility antigens of the A, B, and C HLA regions can be investigated. In the sequencing of MSLA antigens, peptides are formed and can be studied to see if they retain the alloantigenic activity of the intact antigen. Another use for purified MSLA antigens is to insert them in liposomes or phospholipid model membrane systems, to see if they maintain their activity and are able to stimulate mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) reactions.

Therefore, the isolation of miniature swine $\beta_2\mu$ not only provides a purified $\beta_2\mu$ molecule for study and comparison with other species, but also facilitates the purification of MSLA antigens. There are multiple uses for these purified MSLA antigens, all leading to a better understanding of the MHC and its role in transplantation and various immunological functions.

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